Innovative thrombolytic strategy using a heterodimer diabody against TAFI and PAI-1 in mouse models of thrombosis and stroke

Short title: Dual TAFI/PAI-1 inhibitor-enhanced fibrinolysis

Tine Wyseure[1] PhD, Marina Rubio[2] PhD, Frederik Denorme[3] MSc, Sara Martinez de Lizarrondo[2] PhD, Miet Peeters[1], Ann Gils[1] PhD, Simon F. De Meyer[3] PhD, Denis Vivien[2] PhD and Paul J. Declerck[1] PhD.

[1] *Laboratory for Therapeutic and Diagnostic Antibodies, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, Belgium*

[2] *INSERM, INSERM UMR-S U919, Institut National de la Santé Et de la Recherche Médicale - Serine Protease and Pathophysiology of the Neurovascular Unit, University Caen Basse-Normandie, GIP Cyceron, Caen, France*

[3] *Laboratory for Thrombosis Research, KU Leuven Kulak, Kortrijk, Belgium*

Corresponding author:

Paul Declerck
Laboratory for Therapeutic and Diagnostic Antibodies
Department of Pharmaceutical and Pharmacological Sciences
KU Leuven
Campus Gasthuisberg, O&N2, PB 820
Herestraat 49
B-3000 Leuven, Belgium
Phone: +32-16-32 34 31
Fax : +32 16-32 34 60
e-mail:paul.declerck@pharm.kuleuven.be

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Key points

1. Early thrombolytic treatment with a bispecific inhibitor against TAFI and PAI-1 is effective without exogenous tPA
2. Even at the highest dose tested, the bispecific inhibitor against TAFI and PAI-1 does not prolong bleeding time.

Abstract

Circulating thrombin-activatable fibrinolysis inhibitor (TAFI) and plasminogen activator inhibitor-1 (PAI-1) are causal factors for thrombolytic failure. Therefore, we evaluated an antibody-engineered bispecific inhibitor against TAFI and PAI-1 (heterodimer diabody, Db-TCK26D6x33H1F7) in several mouse models of thrombosis and stroke. Prophylactic administration of the diabody (0.8mg/kg) in a thromboplastin-induced model of thromboembolism led to decreased lung fibrin deposition. In a model of cerebral ischemia/reperfusion, diabody administration (0.8mg/kg, 1h post occlusion) led to a mitigated cerebral injury with a 2.3-fold reduced lesion and improved functional outcomes. In a mouse model of thrombin-induced middle cerebral artery occlusion (MCAo), the efficacy of the diabody was compared to the standard thrombolytic treatment with recombinant tissue-type plasminogen activator (tPA). Early administration of diabody (0.8mg/kg, 20min post occlusion) caused a 2-fold decrease in brain lesion size, whereas that of tPA (10mg/kg) had a much smaller effect. Delayed administration of diabody or tPA (90min post occlusion) had no effect on lesion size, whereas the combined administration of diabody with tPA caused a 1.7-fold decrease in lesion size. In contrast to tPA, the diabody did not increase accumulative bleeding. In conclusion, administration of a bispecific inhibitor against TAFI and PAI-1 results in a prominent profibrinolytic effect in mice without increased bleeding.

Introduction

Plasminogen activators are the only thrombolytic agents approved to rapidly revascularize a thrombosed vessel. Reperfusion of the ischemia-affected organ leads to an improved outcome in patients when applied within the first hours after ischemic onset.[1] Despite this evidence-based beneficial effect, current thrombolytic agents remain widely underutilized due to life-threatening side-effects, such as cerebral hemorrhages and possible neurotoxicity.[2,3] For acute ischemic stroke, in particular, the only licensed treatment option consists of a high systemic dose of recombinant tissue-type plasminogen activator (tPA), which is actually given to less than 10% of the patients. Therefore, there is an unmet clinical need to explore novel therapeutic avenues to enhance fibrinolysis without plasminogen activator-associated adverse effects. Endogenous intravascular fibrinolysis is driven upon the release of tPA from the endothelium, which in turn enzymatically activates plasminogen into the fibrin-degrading enzyme, plasmin.[4] This activation step is attenuated by circulating thrombin-activatable fibrinolysis inhibitor (TAFI) and plasminogen activator inhibitor-1 (PAI-1). Activated TAFI (TAFIa, encoded by the *CPB2* gene) eliminates C-terminal Lys exposed on partially degraded fibrin which ultimately leads to a diminished efficiency and localization of plasminogen activation by t-PA.[5] PAI-1 belongs to the serine protease inhibitor (serpin) superfamily and is the primary inhibitor of tPA. The active form of PAI-1 can irreversibly neutralize the activity of its target serine protease by forming a 1:1 stoichiometric covalent complex, accompanied by deformation of the catalytic triad of the serine protease.[6]

Accordingly, elevated plasma levels of TAFI and/or PAI-1 instigate an increased risk for thrombotic disorders (e.g. venous thromboembolism,[7] acute ischemic stroke[8] and acute myocardial infarction[9,10]) and cause thrombolytic failure.[11,12] A considerable number of studies using animal thrombosis models have demonstrated a beneficial effect of TAFI or PAI-1 as drug targets,[13] but no study has yet shown thrombolytic superiority over plasminogen activators.[14,15] We postulate that single targeting of the anti-fibrinolytic system does not suffice for robust thrombolysis. Since TAFI and PAI-1 act

complementary at the fibrin surface,[16] we hypothesized that dual TAFI/PAI-1 targeting would allow efficient thrombolysis at safer systemic drug levels.

Highly specific monoclonal antibodies (MA) against TAFI and PAI-1 are available and have been previously validated *in vivo*.[17-19] MA-TCK26D6 modulates TAFI/TAFIa by impairing activation mediated by thrombin and plasmin, however it does not block the stronger activator, thrombin/thrombomodulin.[17] Interestingly this antibody also prevents the specific interaction of TAFIa on fibrin, but not on the other small pro-inflammatory substrates of TAFIa.[20] MA-33H1F7 converts the active form of PAI-1 to a substrate for tPA, leading to the irreversible cleavage of PAI-1.[21] TAFI and PAI-1 both act in the vicinity of a fibrin clot, making simultaneous targeting of the two antigens through one protein a feasible and potentially safer strategy. Previously, a corresponding small recombinantly engineered bispecific antibody, a diabody, was developed to target TAFI and PAI-1 simultaneously and was designated as Db-TCK26D6x33H1F7.[22] In a thromboelastometric assay, we confirmed that simultaneous inhibition of TAFI and PAI-1 causes an enhancement of *in vitro* fibrinolysis in human and mouse blood.[22]

In the present study, Db-TCK26D6x33H1F7 was used to evaluate the concept of dual TAFI/PAI-1 inhibition in models of thromboembolism and stroke. The thromboprophylactic capacity of the diabody was evaluated in a well-established mouse model of venous thromboembolism.[17,18,23,24] In addition, the effect of the diabody on brain ischemia and reperfusion injury was assessed in a mechanical transient middle cerebral artery occlusion (MCAo) model in which brain lesion and neurological/motor outcome were measured. Furthermore, the profibrinolytic capacity was evaluated in two stroke models where *in situ* clots are induced in the vascular lumen of the middle cerebral artery (MCA) via a thrombin- and a $FeCl_3$-induced MCAo model. The goal was to compare the efficacy of the diabody *vs.* the standard thrombolytic treatment with tPA post thrombus formation. Finally, safety regarding potential bleeding risks was evaluated.

Methods

A detailed description of the methods is provided in the Online Data Supplement.

Animals

Thromboplastin-induced thromboembolism, pharmacokinetics and tail bleeding assay were performed on female SWISS mice (18-22 g, Janvier, France). Mechanical transient middle cerebral artery occlusion (tMCAo) was performed on male/female C57BL/6J mice (16-20 g for females and 20-24 g for male, The Jackson laboratory). Thrombotic middle cerebral artery occlusion (MCAo) was performed on male SWISS mice (30-35 g, Janvier). Experiments were performed in accordance with local ethical laws and the local ethical committees (*cfr.* Online Data supplement for details) and European Communities Council Directives of November 24, 1986 (86/609/EEC) guidelines for the care and use of laboratory animals. All experiments were performed following the ARRIVE guidelines (www.nc3rs.org.uk), including randomization of treatment as well as surgery and analysis blind to the treatment.

Venous thromboembolism model

Thromboplastin-induced thromboembolism was performed as described previously[17] with slight modifications. Endotoxemia was induced in overnight fasted non-anesthetized mice by intraperitoneal injection of lipopolysaccharide (LPS; 0.5mg/kg) from *Escherichia coli* serotype 026:B6 (Sigma-Aldrich, St. Louis, MO, USA) 3 h prior to the start of the experiment. Then, diabody (0.2 mg/kg or 0.8mg/kg) or vehicle (isotonic saline) was intravenously (IV) administered via tail vein injection in endotoxemic mice. Five min post treatment, thromboembolism was induced by IV injection of thromboplastin (corresponding to a dose of 2.5µg/kg tissue factor). Fibrin deposition in lungs was quantified 15 min post thromboplastin injection as described previously (see supplement).[17]

Mechanical transient MCAo (tMCAo)

Surgical procedures and treatments

Mechanical transient MCAo (tMCAo) was performed as described previously according to the monofilament method.[25] The intraluminal suture was left *in situ* during 60 min. Then animals were reanesthetized, and the occluding monofilament was withdrawn to allow reperfusion. Immediately after reperfusion, diabody (0.8mg/kg) or vehicle (PBS) was administered IV.

Neurological tests

24 h post occlusion, mice were subjected to the modified Bederson test[26] and the grip test[27] to assess global neurological function and motor function, respectively (see supplement).

Lesion quantification and cerebral fibrinogen

24 h post occlusion, mice were euthanized to quantify lesion volumes through staining with 2% 2,3,5-triphenyl-tetrazolium chloride. Cerebral fibrin(ogen) was determined as described in the supplemental section "protein extraction and Western blot analysis".

Thrombotic (thrombin-induced) MCAo

Surgical procedure and treatments

Deeply anesthetized mice by inhalation of 5% isoflurane/oxygen mixture were placed on a stereotaxic device and were maintained under anesthesia by inhalation of 2% isoflurane/oxygen mixture during surgical procedures. Body temperature was maintained at 37°C with a thermostat-controlled heating pad throughout the whole procedure. Right middle cerebral artery (MCA) was exposed by craniectomy. *In situ* occlusion of the MCA was performed by micro-injection of murine alpha-thrombin (1 IU, Kordia, Leiden, The Netherlands) into the MCA[28] and time of occlusion was determined by Laser Doppler flowmetry. Diabody (Db) or vehicle (PBS) was injected IV via a tail vein catheter at certain time points post occlusion: an early (20 min), intermediate (90 min) or late (240

min) time point. Five min after diabody or vehicle administration, tPA (10mg/kg) or saline was administered IV (10% as bolus and 90% infused over 40 min).

Magnetic resonance imaging (MRI) analysis

MRI was performed at 24 h post occlusion on a Pharmascan 7 T/12 cm system using surface coils (Bruker Biospin, Wissembourg, France). Brain lesion volume was determined by T2-weighted MRI (Multi-Slice Multi-Echo (MSME) sequence: TR/TE 2500 ms/51 ms[29]), the angiographic scoring of the MCA (0= occlusion, 1= partial recanalization and 2= complete recanalization) was determined by MR angiography (MRA) (2D-TOF sequences [30]) and cerebral hemorrhages were detected by T2*-weighted MRI (Fast Low Angle Shot sequence: TR/TE 350 ms/ 6 ms). Mice with a lesion volume <3 mm^3 were excluded (no mice were excluded from thrombin-induced MCAo experiments).

Tail bleeding assay

Mouse tail vein bleeding times and hemoglobin loss were determined with a tail-clipping assay, as described previously (see supplement).[31]

Statistical analysis

All quantitative data are presented as mean and standard error of mean (SEM). Statistical analysis was performed with GraphPad Prism Version 5 (GraphPad Software) (see supplement). P-values less than 0.05 were considered significant.

Results

Characterization of the profibrinolytic effect of diabody in a model of venous thromboembolism

In a model of thromboplastin-induced thromboembolism, which is well-established for the evaluation of profibrinolytic agents,[17,18,23,32] the diabody was tested in order to determine an effective dosage. Because of the extremely low baseline levels of PAI-1 in mice, endotoxemic mice were used in this type of acute model to obtain a potential contributing effect of PAI-1 inhibition. Previously it was reported that under the given conditions, endotoxemic mice had an increased PAI-1 plasma level of 112±30 ng/mL.[33] In this study, the plasma level of TAFI was also measured under endotoxemic conditions and was found to be 6.5±0.4 µg/mL (n=3). An initial dosage of 0.2 mg/kg was administered IV to obtain a circulating concentration of diabody theoretically equimolar to TAFI [TAFI plasma level (µg/mL) x plasma volume/body weight (mL/mg) x correction Mw: 6.5 µg/mL x 0.030 mL/mg x 58 kDa /56 kDa= 0.2mg/kg]. At 0.2 mg/kg diabody, thromboplastin-induced fibrin deposition in lungs was reduced, however not to a significant degree. With a higher dosage of 0.8 mg/kg diabody, however, fibrin deposition in the lungs was maximally reduced (94% reduction; Figure 1; $p<0.01$ *vs.* vehicle, n=10 mice/group). In fact, 5 min post administration of 0.8 mg/kg diabody, the plasma level was 6.6±0.2 µg/mL diabody (n=3, Supplemental figure S2), which is consistent with the rapid initial clearance ($t_{1/2\alpha}$) of a diabody.[34] Thus, administration of diabody at 0.8 mg/kg results in an approximately equimolar concentration to TAFI shortly after injection. The diabody was used at a dose of 0.8 mg/kg in further experiments, except for the thrombolysis experiments on $FeCl_3$-induced thrombi (Supplemental figure S3), which are more resistant to fibrinolysis.

Profibrinolytic effect of diabody on cerebral ischemia/reperfusion injury

A mechanical tMCAo model was used to assess the effect of diabody on cerebral ischemia/reperfusion injury when administered 1 h post occlusion. This model typically yields large lesion volumes in untreated mice which have measurable neurological/motor defects. Experiments

on the pharmacokinetics of the diabody showed that its circulating half-life ($t_{1/2\beta}$) is 121 min (Supplemental figure S2), which allows its administration by a single bolus. 24 h post occlusion, mice treated with diabody (0.8 mg/kg) exhibited significantly reduced lesion volumes (Figures 2A-B; $p<0.01$; n=10-12 mice/group) and concomitantly improved neurological (Figure 2C; $p<0.05$; n=10-12 mice/group) and motor scores (Figure 2D; $p<0.01$; n=10-12 mice/group). In addition, western blot analysis revealed that the diabody effectively reduced massive fibrin deposition induced by reperfusion injury by at least 2-fold (Figures 2E-F; $p<0.05$; n=4 mice/group).

Thrombolytic effect of the diabody on fibrin-rich clots in a model of thrombin-induced stroke

A model of thromboembolic stroke by local thrombin injection was used in which clots are rich in fibrin and thus susceptible to be thrombolysed by tPA when administered early post clot onset, i.e. 20 min post occlusion. The efficacy of the diabody was compared to that of tPA, the current thrombolytic agent. In order to mimic the clinical procedure of thrombolysis, the administration of tPA was performed by an initial bolus of 10% volume followed by 90% infusion during 40 min because of the short half-life of circulating tPA (~ 5 min[35]). 24 h post occlusion, complete recanalization of the arterial lumen occurred in all groups including the vehicle group (median angiographic score=2). Lesion volume was reduced by early administration of tPA, however this reduction was not statistically significant (37±13 mm^3 *vs.* 26±12 mm^3; Figure 3A; $p=0.203$; n=6-8 mice/group). In contrast, early diabody administration (0.8 mg/kg), regardless of the co-administration of tPA, substantially reduced lesion volume at 24 h (15±4 mm^3 for diabody and 15±8 mm^3 for diabody + tPA; $p<0.01$ and $p<0.05$ *vs.* vehicle respectively; n=6-8 mice/group; Figure 3A).

When delaying the treatments to a clinically more relevant time point, e.g. 90 min post occlusion (intermediate time point),[36] complete recanalization was also observed at 24 h post occlusion in all treatment groups (median angiographic score= 2). Intermediately delayed administration of diabody nor infusion of tPA had any beneficial effect on the lesion volume (25±3 mm^3 (vehicle) *vs.* 24±3 mm^3 (tPA) *vs.* 21±4 mm^3 (Db); Figure 3B; n=9-10 mice/group). However, at the same treatment time point

diabody administration prior to tPA infusion resulted in a significantly reduced lesion volume (15±2 mm^3 (Db+tPA); $p<0.05$ *vs.* vehicle; n=10 mice/group; Figure 3B).

None of the treatments had an effect on lesion sizes in this model when administered at 240 min post occlusion (late time point, Figure 3C).

Assessment of bleeding

Tail bleeding experiments were performed to compare the effects of an IV injection of tPA at two different doses: the dose equivalent to that used in clinical practice for humans (1 mg/kg) and to that typically used in mice (10 mg/kg). Diabody was injected at 0.8 mg/kg and 3.6 mg/kg. IV administration of diabody up to 3.6 mg/kg did not alter tail bleeding time or accumulative hemoglobin loss after 60 min tail incubation, whereas both doses of tPA prolonged bleeding time and increased hemoglobin loss (Figure 4A-B; n=9-10 mice/group). Co-administration of diabody (0.8 mg/kg) and tPA (10 mg/kg), the treatment regimen tested in the thrombin-mediated MCAo model, did not further increase the tail bleeding time nor hemoglobin loss compared to tPA administration alone.

Alternatively, no cerebral hemorrhages were observed in either mechanical or thrombotic MCAo stroke models after any treatment.

Discussion

Based on a considerable amount of clinical data, elevated levels of antifibrinolytic proteins are linked to a potentiated risk for intravascular thrombosis, recurrent thrombosis and thrombolytic failure.[7,8,10,11] Antifibrinolytic proteins, such as TAFI and PAI-1, have been extensively studied as drug targets in thrombosis-related pathologies.[13] Notwithstanding promising results, these studies altogether do not conclusively proclaim that inhibition of one antifibrinolytic constituent is sufficient to overcome thrombosis.[31,37,38] Since TAFI and PAI-1 have complementary roles that are localized at the fibrin surface, we hypothesized that simultaneous targeting of the two antifibrinolytics would amplify the profibrinolytic capacity in a fibrin localized manner. Fibrinolysis is driven by enzymatic activation of plasminogen into active plasmin through the exposure of C-terminal lysines subsequent to plasmin-mediated cleavage of fibrin.[4] Since these C-terminal lysines serve as co-factors for tPA-mediated activation of plasminogen, this results in a self-propagating dissolution of fibrin. Through the enzymatic removal of C-terminal lysines from fibrin, TAFIa thereby confines fibrinolysis. TAFIa, however, prevents fibrinolysis from entering into the propagation phase only when above a certain threshold concentration which is proportionate to the level of plasmin activity, i.e. a higher level of plasmin generation requires a higher amount of generated TAFIa to withhold fibrinolysis. In terms of a profibrinolytic strategy, a PAI-1 inhibitor leads to an increased t-PA activity resulting in more plasmin generation and therefore causing an elevated threshold concentration of TAFIa. The latter in combination with a TAFI inhibitor results in a strong profibrinolytic effect, i.e. fibrinolysis occurs in the presence of much lower concentrations of t-PA.

To facilitate drug development a two-in-one molecule was designed, called a diabody, to target TAFI and PAI-1 simultaneously. An additional advantage of such diabody construct was previously exemplified *in vitro* with Db-T12D11x33H1F7[39] and Db-TCK26D6x33H1F7.[22] Db-TCK26D6x33H1F7 was specifically generated for the species cross-reactive properties of the parental antibodies: besides its cross-reactivity towards human TAFI and human PAI-1 allowing its potential clinical use, the diabody can also be evaluated in preclinical studies (without the need of a surrogate diabody). In

both *in vitro* studies, the diabodies were more efficient in increasing fibrinolysis than the combined use of the corresponding parental antibodies. The increased *in vitro* potency is most likely the consequence of the smaller (58 kDa) and more flexible structure of a diabody, favoring efficient penetration and diffusion into the blood clot. The current study describes the first use of such profibrinolytic strategy *in vivo*. Db-TCK26D6x33H1F7 was first applied thromboprophylactically in a well-established mouse model of thromboplastin-induced thromboembolism in which a low dose of 0.8 mg/kg (resulting in an equimolar plasma concentration of TAFI) was maximally effective in clearing fibrin from lungs (Figure 1).

A common phenomenon in stroke patients is secondary infarct growth despite initial vessel recanalization. The precise mechanisms of this so called 'reperfusion injury' are still not completely understood but involve complex thrombo-inflammatory processes. Intravascular fibrin deposition, trapping erythrocytes, leukocytes and platelets have shown to directly obstruct cerebral microvascular perfusion during ischemia and reperfusion.[40-43] To further assess the potential profibrinolytic effect of the diabody in this setting, it was evaluated in a mechanical tMCAo model of cerebral ischemia/reperfusion injury. The diabody prominently reduced fibrin(ogen) deposition and lesion volume, which was associated with improved neurological and motor outcome at 24 h (Figure 2). In line with this observation, TAFI and PAI-1 levels are increased in patients suffering from ischemic stroke, often correlating with stroke severity, suggesting a negative impact of these anti-fibrinolytic molecules in stroke development.[8,44-50] Interestingly, whereas the diabody ameliorated the outcome in this model, tPA has a controversial effect, potentially through aggravating neuronal damage after focal cerebral ischemia.[51] As a control, an *in vitro* neurotoxicity assay was performed with or without N-methyl-D-aspartate (NMDA) and demonstrated the absence of any neurotoxicity associated with the diabody (Supplemental figure S4).

Because there is an unmet clinical need to optimize current thrombolytic treatment of acute ischemic stroke, we next focused on mouse models of thrombotic MCAo. Current treatment of acute

ischemic stroke consists of a high dose of tPA which is only effective within 4.5 h after symptom onset, however only 20% of the small number of patients which arrive in time receive thrombolytic therapy.[2,3] This reluctance against thrombolysis stems from life-threatening side effects (hemorrhagic transformation and possible neurotoxicity of tPA) while the thrombus resolving efficiency of tPA is rather low.[52,53] Unexpectedly, neuroprotective agents, which block molecular elaboration of ischemic insult on brain cells, have shown no clinical benefit in patients despite their preclinical efficacy.[54] Since recanalization of the occluded vessel is in essence associated with improved clinical outcome,[1] it makes sense to target proteins, such as TAFI and PAI-1, that inhibit thrombolysis.[11] Indeed, when the diabody (0.8 mg/kg) was administered early after thrombin-mediated MCAo, a strong beneficial effect on lesion volume was observed (Figures 3A). This reduction of lesion volume even exceeded the effect of a high systemic dose of tPA (10 mg/kg). To the best of our knowledge, this is the first report of a pharmacological treatment of fibrin-rich clots resulting in a more efficient lysis than with systemic administration of plasminogen activators.

According to a meta-analysis of ATLANTIS, ECASS, and NINDS rt-PA stroke trials, the greatest benefit is observed if tPA treatment is initiated within 90 min post stroke onset.[36] At 90 min post stroke onset and onwards, tPA treatment does not always result in a beneficial outcome, presumably because of the increased stability of the clot (i.e. clot retraction) resulting in thrombolytic resistance, the neurotoxic effect of tPA to the progressively damaged brain and/or the increased risk for hemorrhagic transformation. Therefore in the current study, 90 min post occlusion was tested in the thrombin-mediated MCAo model (Figure 3B). In contrast to the earlier treatment time point (i.e. 20 min post occlusion), tPA treatment nor diabody treatment at 90 min post occlusion had a beneficial effect on the lesion volumes. Interestingly, the combined treatment of the diabody and tPA resulted in a significantly decreased lesion volume, underscoring the potential clinical benefit of adding the diabody to current thrombolytic treatment. Furthermore, a later treatment time point of 4 h post occlusion was tested at which tPA has previously been established to have a deleterious effect (i.e. increased lesion volume). In our hands, however, we only observed a tendency towards increased

lesion volumes after tPA treatment, alone or with diabody (Figure 3C). Diabody treatment at this time point did not have any effect, which is in line with the spontaneous resolution of the thrombin-induced clot in this model.[55] In correspondence to the *in vitro* neurotoxicity data (Supplemental figure S4), the diabody also had no deleterious effect *in vivo*.

To address another clinically relevant issue in the thrombolytic treatment of stroke, a $FeCl_3$-induced MCAo model was used in which platelet-rich clots are formed that are resistant to tPA. The presence of resistant platelet-rich clots contributes to the failure of thrombolytic therapy in the majority of patients.[52,53] Thus, in this model, tPA does not reduce the lesion volume nor does it improve recanalization, even when administered early i.e. 20 min post occlusion.[29] In clinical practice, cerebral blood flow (CBF) is often measured during and early after tPA administration to evaluate the success of tPA-mediated thrombolysis.[56] For this reason in this model, CBF of the MCA was monitored by Laser Doppler flowmetry up to 1 h after stroke onset in this set of experiments. In addition, recanalization of the MCA was determined by MRA at 24 h. As expected no increase in either CBF or recanalization rate was observed with vehicle or exogenous tPA, whereas the diabody (at 1.6 mg/kg and 3.6 mg/kg) effectuated a slight, but significant increase of CBF at 1 h which further led to an increased recanalization rate at 24 h (Supplemental figure S3). Although no difference was detected between both dosages of diabody in terms of CBF and recanalization rate, only the higher dose was able to decrease lesion volumes.

Bleeding is a feared complication of thrombolysis, especially in the cerebral circulation. In the thrombosis-induced stroke models no cerebral bleeding was observed by MRI. However, these models are not suitable for excluding possible cerebral bleeding, since even after tPA administration no hemorrhagic transformations occurred. Cerebral bleeding tendency is related to the lesion volume, which is relatively small in these models. The monofilament however blocks the whole MCA-perfused territory, which typically yields large lesions and more easily transforms into cerebral

bleeding. In this model, rtPA has been previously demonstrated to increase the incidence of bleeding and oedema[57], whereas the diabody in this study did not.

To study the risk of systemic bleeding upon traumatic injury, we performed a tail bleeding model in which tPA and diabody were compared (Figure 4). A similar prolongation of bleeding time upon administration of tPA was observed as in a previous study.[58] The diabody however caused no bleeding time prolongation nor increased accumulative hemoglobin loss. This is in agreement with a previous study in which TAFI/PAI-1 double deficient mice showed no prolonged bleeding.[31] With TAFI and PAI-1 inhibition, the endogenous tPA activity is increased. However, this most likely does not exceed the systemic antifibrinolytic capacity of α_2-antiplasmin. In case of potential side effects, the relatively short half-life of the diabody is also advantageous. However, it is still long enough to allow a bolus administration to obtain sufficiently high levels for several hours.

In conclusion, we have created a strong fibrinolytic enhancer, designated as Db-TCK26D6x33H1F7, with a robust *in vivo* performance in a variety of thrombotic and stroke mouse models. Our results show that the diabody has potential clinical applications in the treatment of thrombotic disorders: (i) prophylaxis of venous thromboembolism, (ii) treatment of brain ischemia/reperfusion injury and (iii) in the thrombolytic treatment of both fibrin-rich and platelet-rich clots in ischemic stroke. This novel profibrinolytic approach appears to be potentially safer and more effective in mouse stroke models than the current treatment with tPA. Based on these results, this diabody holds promise as a candidate for clinical studies of thrombotic disorders.

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Authorship contributions

T.W. designed and performed research, analyzed and interpreted the data, performed statistical analysis, and drafted the manuscript; M.R., F.D. and S.M.D.L. performed research and analyzed the data. M.P. provided technical assistance. A.G. provided scientific suggestions and contributed to manuscript review; S.F.D.M. and D.V. contributed to the design of this study and to manuscript review. P.J.D. conceived and designed the study, coordinated the experiments and reviewed the manuscript.

All authors read and approved the final manuscript.

Disclosure of Conflicts of Interest

A patent application has been filed to protect the intellectual property of the described diabody.

References

1. Heiss WD, Grond M, Thiel A, et al. Tissue at risk of infarction rescued by early reperfusion: a positron emission tomography study in systemic recombinant tissue plasminogen activator thrombolysis of acute stroke. *J Cereb Blood Flow Metab*. 1998;18(12):1298-1307.
2. Eissa A, Krass I, Bajorek BV. Optimizing the management of acute ischaemic stroke: a review of the utilization of intravenous recombinant tissue plasminogen activator (tPA). *J Clin Pharm Ther*. 2012;37(6):620-629.
3. Dávalos A. Thrombolysis in acute ischemic stroke: successes, failures, and new hopes. *Cerebrovasc Dis*. 2005;20 Suppl 2:135-139.
4. Rijken DC, Lijnen HR. New insights into the molecular mechanisms of the fibrinolytic system. *J Thromb Haemost*. 2009;7(1):4-13.
5. Bajzar L, Manuel R, Nesheim ME. Purification and characterization of TAFI, a thrombin-activable fibrinolysis inhibitor. *J Biol Chem*. 1995;270(24):14477-14484.
6. Huntington JA, Read RJ, Carrell RW. Structure of a serpin-protease complex shows inhibition by deformation. *Nature*. 2000;407(6806):923-926.
7. Meltzer ME, Lisman T, de Groot PG, et al. Venous thrombosis risk associated with plasma hypofibrinolysis is explained by elevated plasma levels of TAFI and PAI-1. *Blood*. 2010;116(1):113-121.
8. Leebeek FW, Goor MP, Guimaraes AH, et al. High functional levels of thrombin-activatable fibrinolysis inhibitor are associated with an increased risk of first ischemic stroke. *J Thromb Haemost*. 2005;3(10):2211-2218.
9. Vaughan DE, Rouleau JL, Ridker PM, Arnold JM, Menapace FJ, Pfeffer MA. Effects of ramipril on plasma fibrinolytic balance in patients with acute anterior myocardial infarction. HEART Study Investigators. *Circulation*. 1997;96(2):442-447.
10. Thögersen AM, Jansson JH, Boman K, et al. High plasminogen activator inhibitor and tissue plasminogen activator levels in plasma precede a first acute myocardial infarction in both men and women: evidence for the fibrinolytic system as an independent primary risk factor. *Circulation*. 1998;98(21):2241-2247.
11. Fernandez-Cadenas I, Alvarez-Sabin J, Ribo M, et al. Influence of thrombin-activatable fibrinolysis inhibitor and plasminogen activator inhibitor-1 gene polymorphisms on tissue-type plasminogen activator-induced recanalization in ischemic stroke patients. *J Thromb Haemost*. 2007;5(9):1862-1868.
12. Brouns R, Heylen E, Sheorajpanday R, et al. Carboxypeptidase U (TAFIa) decreases the efficacy of thrombolytic therapy in ischemic stroke patients. *Clin Neurol Neurosurg*. 2009;111(2):165-170.
13. Wyseure T, Declerck PJ. Novel or expanding current targets in fibrinolysis. *Drug Discov Today*. 2014;19(9):1476-1482.
14. Izuhara Y, Yamaoka N, Kodama H, et al. A novel inhibitor of plasminogen activator inhibitor-1 provides antithrombotic benefits devoid of bleeding effect in nonhuman primates. *J Cereb Blood Flow Metab*. 2010;30(5):904-912.
15. Wang YX, da Cunha V, Vincelette J, et al. A novel inhibitor of activated thrombin activatable fibrinolysis inhibitor (TAFIa) - part II: enhancement of both exogenous and endogenous fibrinolysis in animal models of thrombosis. *Thromb Haemost*. 2007;97(1):54-61.
16. Mutch NJ, Thomas L, Moore NR, Lisiak KM, Booth NA. TAFIa, PAI-1 and alpha-antiplasmin: complementary roles in regulating lysis of thrombi and plasma clots. *J Thromb Haemost*. 2007;5(4):812-817.
17. Vercauteren E, Emmerechts J, Peeters M, Hoylaerts MF, Declerck PJ, Gils A. Evaluation of the profibrinolytic properties of an anti-TAFI monoclonal antibody in a mouse thromboembolism model. *Blood*. 2011;117(17):4615-4622.

18. Van De Craen B, Scroyen I, Abdelnabi R, et al. Characterization of a panel of monoclonal antibodies toward mouse PAI-1 that exert a significant profibrinolytic effect in vivo. *Thromb Res*. 2011;128(1):68-76.
19. Berry CN, Lunven C, Lechaire I, Girardot C, O'Connor SE. Antithrombotic activity of a monoclonal antibody inducing the substrate form of plasminogen activator inhibitor type 1 in rat models of venous and arterial thrombosis. *Br J Pharmacol*. 1998;125(1):29-34.
20. Semeraro F, Ammollo CT, Gils A, Declerck PJ, Colucci M. Monoclonal antibodies targeting the antifibrinolytic activity of activated TAFI but not the anti-inflammatory activity on osteopontin and C5a. *J Thromb Haemost*. 2013;11(12):2137-2147.
21. Debrock S, Declerck PJ. Neutralization of plasminogen activator inhibitor-1 inhibitory properties: identification of two different mechanisms. *Biochim Biophys Acta*. 1997;1337(2):257-266.
22. Wyseure T, Gils A, Declerck PJ. Evaluation of the profibrinolytic properties of a bispecific antibody-based inhibitor against human and mouse thrombin-activatable fibrinolysis inhibitor and plasminogen activator inhibitor-1. *J Thromb Haemost*. 2013;11(11):2069-2071.
23. Hendrickx ML, Zatloukalova M, Hassanzadeh-Ghassabeh G, Muyldermans S, Gils A, Declerck PJ. Identification of a novel, nanobody-induced, mechanism of TAFI inactivation and its in vivo application. *J Thromb Haemost*. 2013;12(2):229-236.
24. Hendrickx ML, Zatloukalova M, Hassanzadeh-Ghassabeh G, Muyldermans S, Gils A, Declerck PJ. In vitro and in vivo characterisation of the profibrinolytic effect of an inhibitory anti-rat TAFI nanobody. *Thromb Haemost*. 2014;111(5):824-832.
25. Kleinschnitz C, De Meyer SF, Schwarz T, et al. Deficiency of von Willebrand factor protects mice from ischemic stroke. *Blood*. 2009;113(15):3600-3603.
26. Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. *Stroke*. 1986;17(3):472-476.
27. Moran PM, Higgins LS, Cordell B, Moser PC. Age-related learning deficits in transgenic mice expressing the 751-amino acid isoform of human beta-amyloid precursor protein. *Proc Natl Acad Sci U S A*. 1995;92(12):5341-5345.
28. Orset C, Macrez R, Young AR, et al. Mouse model of in situ thromboembolic stroke and reperfusion. *Stroke*. 2007;38(10):2771-2778.
29. Le Behot A, Gauberti M, Martinez de Lizarrondo S, et al. GpIbα-VWF blockade restores vessel patency by dissolving platelet aggregates formed under very high shear rate in mice. *Blood*. 2014; 123(21):3354-3363.
30. Gauberti M, Montagne A, Marcos-Contreras OA, Le Béhot A, Maubert E, Vivien D. Ultra-sensitive molecular MRI of vascular cell adhesion molecule-1 reveals a dynamic inflammatory penumbra after strokes. *Stroke*. 2013;44(7):1988-1996.
31. Vercauteren E, Peeters M, Hoylaerts MF, et al. The hyperfibrinolytic state of mice with combined TAFI and PAI-1 gene deficiency is critically dependent on TAFI deficiency. *J Thromb Haemost*. 2012;10(12):2555-2562.
32. Muto Y, Suzuki K, Sato E, Ishii H. Carboxypeptidase B inhibitors reduce tissue factor-induced renal microthrombi in rats. *Eur J Pharmacol*. 2003;461(2-3):181-189.
33. Van De Craen B, Scroyen I, Vranckx C, et al. Maximal PAI-1 inhibition in vivo requires neutralizing antibodies that recognize and inhibit glycosylated PAI-1. *Thromb Res*. 2012;129(4):e126-133.
34. Kipriyanov SM, Moldenhauer G, Schuhmacher J, et al. Bispecific tandem diabody for tumor therapy with improved antigen binding and pharmacokinetics. *J Mol Biol*. 1999;293(1):41-56.
35. Chandler WL, Alessi MC, Aillaud MF, Henderson P, Vague P, Juhan-Vague I. Clearance of tissue plasminogen activator (TPA) and TPA/plasminogen activator inhibitor type 1 (PAI-1) complex: relationship to elevated TPA antigen in patients with high PAI-1 activity levels. *Circulation*. 1997;96(3):761-768.
36. Hacke W, Donnan G, Fieschi C, et al. Association of outcome with early stroke treatment: pooled analysis of ATLANTIS, ECASS, and NINDS rt-PA stroke trials. *Lancet*. 2004;363(9411):768-774.

37. Kraft P, Schwarz T, Meijers JC, Stoll G, Kleinschnitz C. Thrombin-activatable fibrinolysis inhibitor (TAFI) deficient mice are susceptible to intracerebral thrombosis and ischemic stroke. *PLoS One*. 2010;5(7):e11658.
38. Nagashima M, Yin ZF, Zhao L, et al. Thrombin-activatable fibrinolysis inhibitor (TAFI) deficiency is compatible with murine life. *J Clin Invest*. 2002;109(1):101-110.
39. Develter J, Booth NA, Declerck PJ, Gils A. Bispecific targeting of thrombin activatable fibrinolysis inhibitor and plasminogen activator inhibitor-1 by a heterodimer diabody. *J Thromb Haemost*. 2008;6(11):1884-1891.
40. Zhang ZG, Chopp M, Goussev A, et al. Cerebral microvascular obstruction by fibrin is associated with upregulation of PAI-1 acutely after onset of focal embolic ischemia in rats. *J Neurosci*. 1999;19(24):10898-10907.
41. Heye N, Cervos-Navarro J. Microthromboemboli in acute infarcts: analysis of 40 autopsy cases. *Stroke*. 1996;27(3):431-434.
42. Okada Y, Copeland BR, Fitridge R, Koziol JA, del Zoppo GJ. Fibrin contributes to microvascular obstructions and parenchymal changes during early focal cerebral ischemia and reperfusion. *Stroke*. 1994;25(9):1847-1853; discussion 1853-1844.
43. del Zoppo GJ, Mabuchi T. Cerebral microvessel responses to focal ischemia. *J Cereb Blood Flow Metab*. 2003;23(8):879-894.
44. Santamaría A, Oliver A, Borrell M, et al. Risk of ischemic stroke associated with functional thrombin-activatable fibrinolysis inhibitor plasma levels. *Stroke*. 2003;34(10):2387-2391.
45. Rooth E, Wallen H, Antovic A, et al. Thrombin activatable fibrinolysis inhibitor and its relationship to fibrinolysis and inflammation during the acute and convalescent phase of ischemic stroke. *Blood Coagul Fibrinolysis*. 2007;18(4):365-370.
46. de Bruijne EL, Gils A, Guimarães AH, et al. The role of thrombin activatable fibrinolysis inhibitor in arterial thrombosis at a young age: the ATTAC study. *J Thromb Haemost*. 2009;7(6):919-927.
47. Kristensen B, Malm J, Nilsson TK, Hultdin J, Carlberg B, Olsson T. Increased fibrinogen levels and acquired hypofibrinolysis in young adults with ischemic stroke. *Stroke*. 1998;29(11):2261-2267.
48. Lindgren A, Lindoff C, Norrving B, Astedt B, Johansson BB. Tissue plasminogen activator and plasminogen activator inhibitor-1 in stroke patients. *Stroke*. 1996;27(6):1066-1071.
49. Catto AJ, Carter AM, Stickland M, Bamford JM, Davies JA, Grant PJ. Plasminogen activator inhibitor-1 (PAI-1) 4G/5G promoter polymorphism and levels in subjects with cerebrovascular disease. *Thromb Haemost*. 1997;77(4):730-734.
50. Margaglione M, Di Minno G, Grandone E, et al. Abnormally high circulation levels of tissue plasminogen activator and plasminogen activator inhibitor-1 in patients with a history of ischemic stroke. *Arterioscler Thromb*. 1994;14(11):1741-1745.
51. Wang YF, Tsirka SE, Strickland S, Stieg PE, Soriano SG, Lipton SA. Tissue plasminogen activator (tPA) increases neuronal damage after focal cerebral ischemia in wild-type and tPA-deficient mice. *Nat Med*. 1998;4(2):228-231.
52. Kim EY, Heo JH, Lee SK, et al. Prediction of thrombolytic efficacy in acute ischemic stroke using thin-section noncontrast CT. *Neurology*. 2006;67(10):1846-1848.
53. Vivien D, Gauberti M, Montagne A, Defer G, Touzé E. Impact of tissue plasminogen activator on the neurovascular unit: from clinical data to experimental evidence. *J Cereb Blood Flow Metab*. 2011;31(11):2119-2134.
54. Dirnagl U, Endres M. Found in Translation: Preclinical Stroke Research Predicts Human Pathophysiology, Clinical Phenotypes, and Therapeutic Outcomes. *Stroke*. 2014.
55. Durand A, Chauveau F, Cho TH, et al. Spontaneous reperfusion after in situ thromboembolic stroke in mice. *PLoS One*. 2012;7(11):e50083.
56. Ribo M, Alvarez-Sabín J, Montaner J, et al. Temporal profile of recanalization after intravenous tissue plasminogen activator: selecting patients for rescue reperfusion techniques. *Stroke*. 2006;37(4):1000-1004.

57. Lapergue B, Dang BQ, Desilles JP, et al. High-density lipoprotein-based therapy reduces the hemorrhagic complications associated with tissue plasminogen activator treatment in experimental stroke. *Stroke*. 2013;44(3):699-707.
58. Suzuki Y, Nagai N, Collen D. Comparative effects of microplasmin and tissue-type plasminogen activator (tPA) on cerebral hemorrhage in a middle cerebral artery occlusion model in mice. *J Thromb Haemost*. 2004;2(9):1617-1621.

Figure legends

Figure 1: Thromboprophylactic effect of the diabody in a model of venous thromboembolism

Fibrin deposition in lungs from endotoxemic mice (n=6-10). Fibrin deposition is expressed by fibrinogen equivalents (µg/ml). Baseline levels were obtained by isolating lungs from healthy mice (n=5) without thrombotic challenge.
The data are represented as mean ± SEM. Statistical significance is indicated by ** ($p< 0.01$). Db indicates diabody.

Figure 2: Evaluation of the diabody (Db) in a model of cerebral ischemia (60 min) and reperfusion.

(A) Representative TTC-stained brain slices; (B) lesion volume (mm^3); (C) Bederson score; (D) Grip test score of mice treated with vehicle or Db (0.8 mg/kg) alone upon reperfusion (60 min post occlusion); (E) representative immunoblots of ipsi- *vs.* contralateral hemispheres and (F) fibrin(ogen) contents in ipsilateral hemisphere (fold increase *vs.* contralateral hemisphere) of mice treated with vehicle or Db (0.8 mg/kg) upon reperfusion (60 min post occlusion). All parameters were measured 24 h post stroke onset. Data are represented as mean ± SEM (B, F) or median (C, D); n=10-12 mice/group (B, C, D); n=4 mice/group (F); *, $p< 0.05$;**, $p< 0.01$. Db indicates diabody.

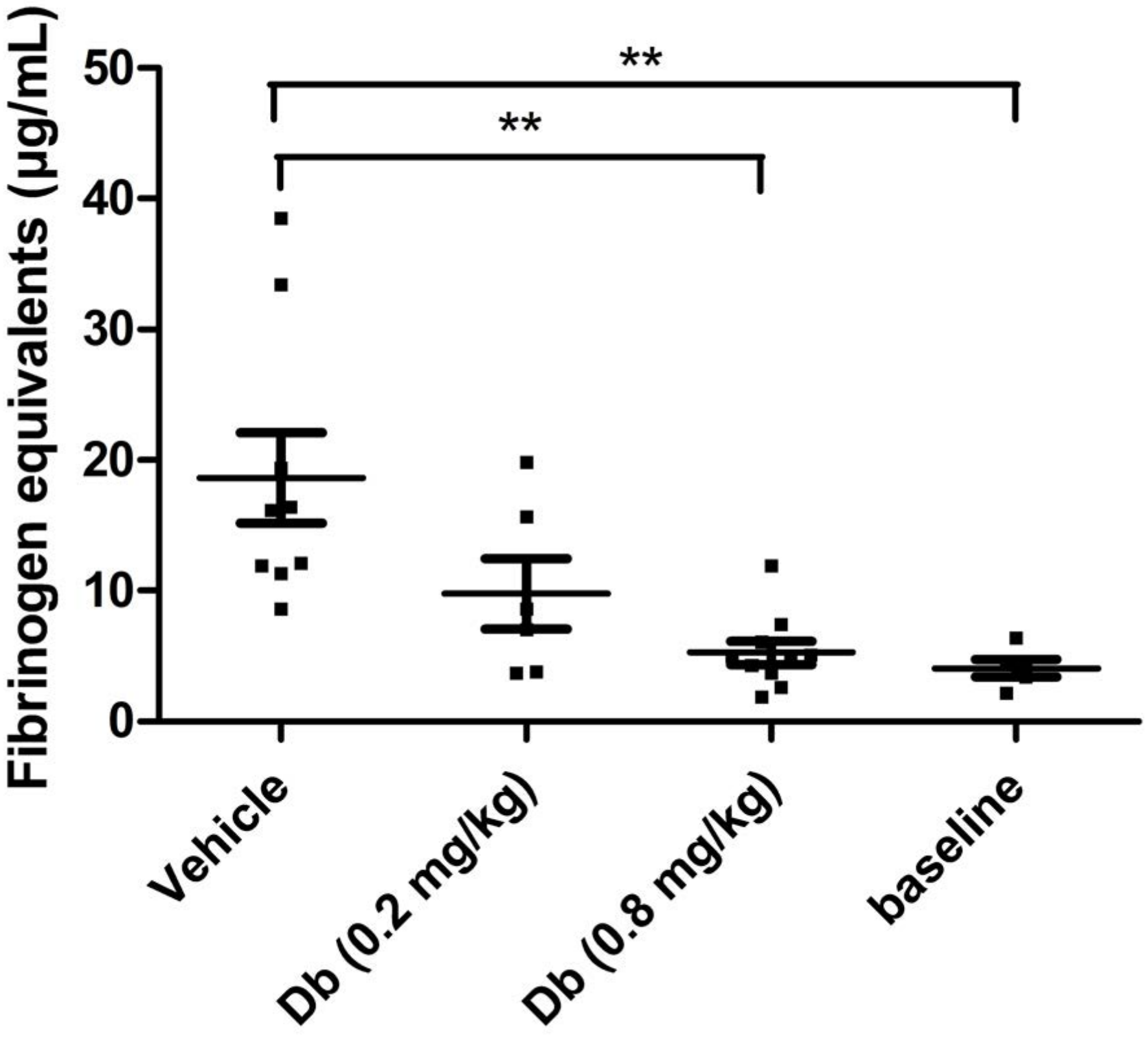
Figure 3: Evaluation of the thrombolytic efficacy of the diabody (Db) on fibrin-rich clots in a model of thrombin-induced MCAo.

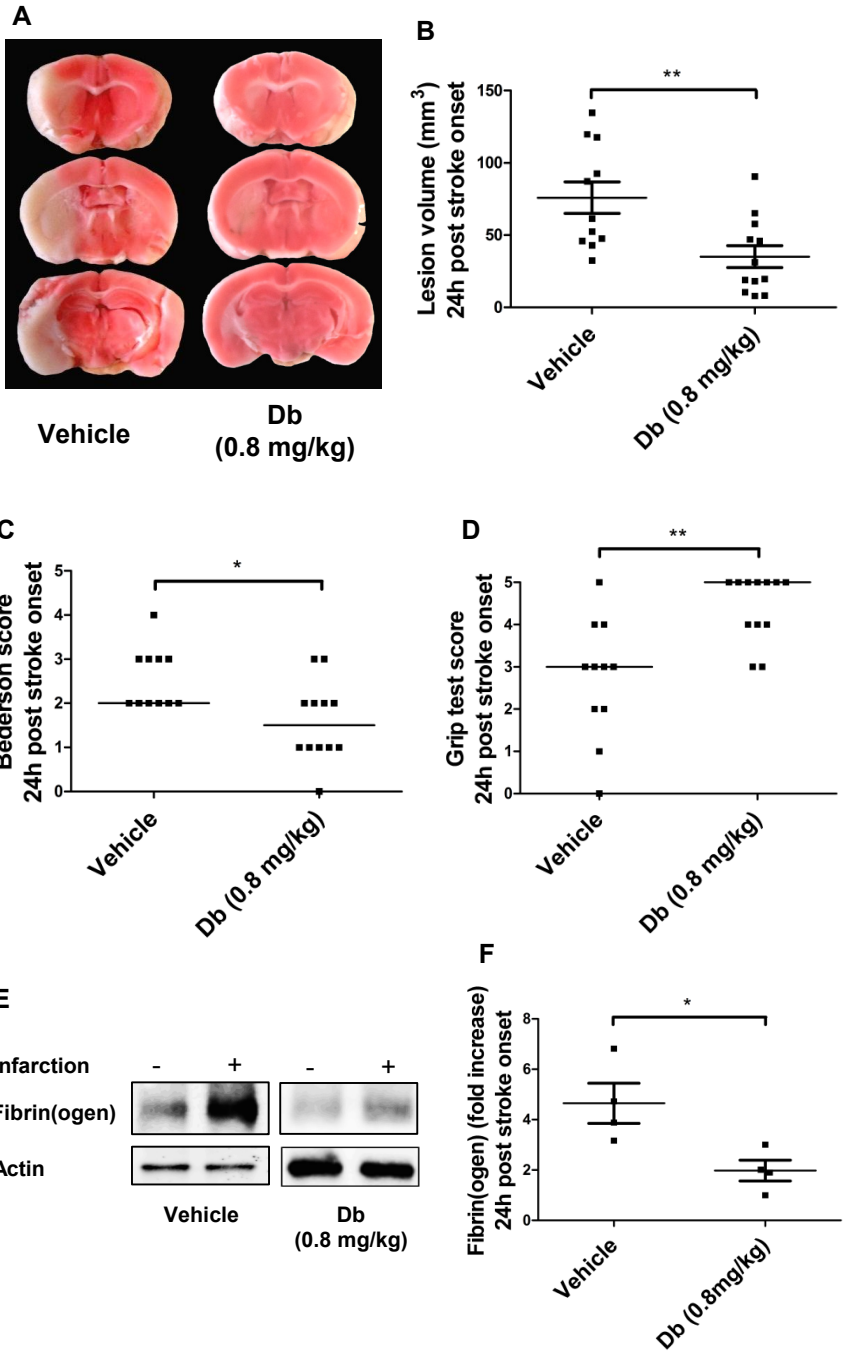
Lesion volume (mm^3) at 24 h post occlusion (top) and representative T2-weighted images 24 h post occlusion (bottom) of mice treated with vehicle, tPA (10 mg/kg), Db (0.8 mg/kg) or a combination of Db (0.8 mg/kg) and tPA (10 mg/kg) 20 min post occlusion (A, n= 6-8), 90 min post occlusion (B, n= 9-10) and 240 min post occlusion (C, n= 7-9). Dotted lines delineate stroke lesions. Data are represented as mean ± SEM. *, $p< 0.05$; **, $p< 0.01$; ns= not significant. tPA indicates recombinant tissue-type plasminogen activator; Db, diabody.

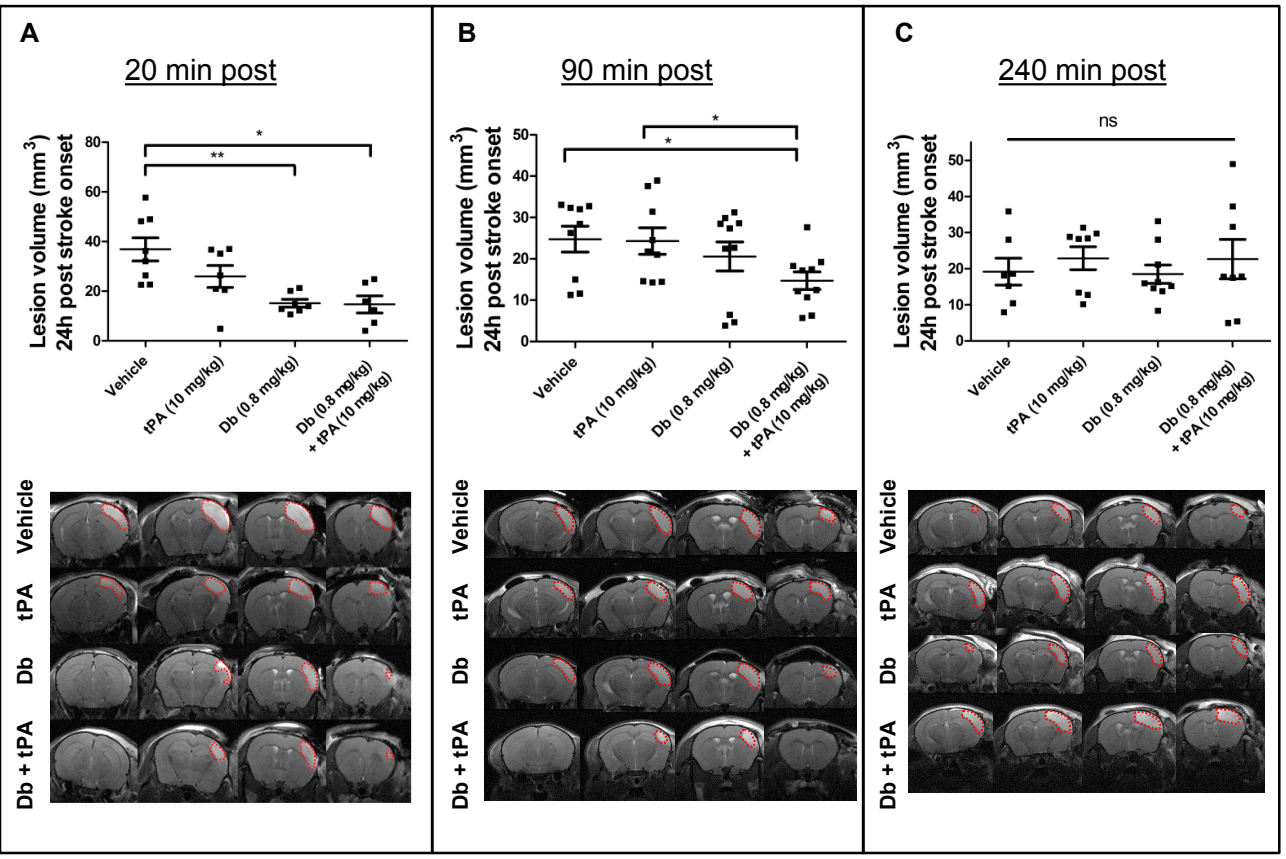
Figure 4: Tail bleeding time and accumulative hemoglobin loss after IV diabody, tPA or diabody + tPA.

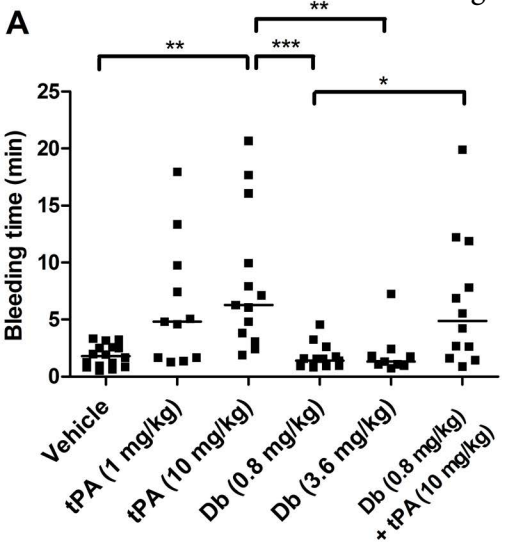
(A) Time (min) till initial cessation of tail bleeding was monitored in mice and (B) accumulative bleeding up to 60 min, measured as hemoglobin (g/dL) (median, n= 9-16 mice/group; *, $p< 0.05$; ** $p< 0.01$; *** $p< 0.005$). tPA indicates recombinant tissue-type plasminogen activator; Db, diabody.

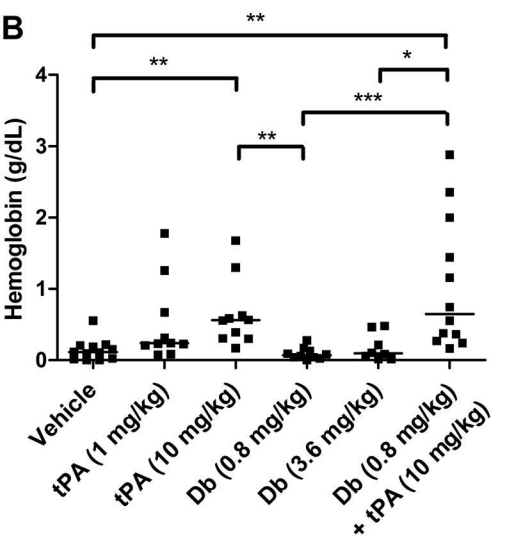
Figure 1


Fibrinogen equivalents (µg/mL)
50
40
30
20
10
0
**
**
Vehicle
Db (0.2 mg/kg)
Db (0.8 mg/kg)
baseline


A
Vehicle
Db
(0.8 mg/kg)
B
Lesion volume (mm3)
24h post stroke onset
150
100
50
0
**
Vehicle
Db (0.8 mg/kg)
C
Bederson score
24h post stroke onset
5
4
3
2
1
0
*
Vehicle
Db (0.8 mg/kg)
D
Grip test score
24h post stroke onset
5
4
3
2
1
0
**
Vehicle
Db (0.8 mg/kg)
E
Infarction
-
+
-
+
Fibrin(ogen)
Actin
Vehicle
Db
(0.8 mg/kg)
F
Fibrin(ogen) (fold increase)
24h post stroke onset
8
6
4
2
0
*
Vehicle
Db (0.8mg/kg)


A
20 min post
Lesion volume (mm3) 24h post stroke onset
80
60
40
20
0
*
**
Vehicle
tPA (10 mg/kg)
Db (0.8 mg/kg)
Db (0.8 mg/kg) + tPA (10 mg/kg)
B
90 min post
Lesion volume (mm3) 24h post stroke onset
50
40
30
20
10
0
*
*
Vehicle
tPA (10 mg/kg)
Db (0.8 mg/kg)
Db (0.8 mg/kg) + tPA (10 mg/kg)
C
240 min post
Lesion volume (mm3) 24h post stroke onset
ns
50
40
30
20
10
0
Vehicle
tPA (10 mg/kg)
Db (0.8 mg/kg)
Db (0.8 mg/kg) + tPA (10 mg/kg)
Vehicle
tPA
Db
Db + tPA


A
**
***
**
*
Bleeding time (min)
25
20
15
10
5
0
Vehicle
tPA (1 mg/kg)
tPA (10 mg/kg)
Db (0.8 mg/kg)
Db (3.6 mg/kg)
Db (0.8 mg/kg) + tPA (10 mg/kg)


B
**
**
*
***
**
Hemoglobin (g/dL)
4
3
2
1
0
Vehicle
tPA (1 mg/kg)
tPA (10 mg/kg)
Db (0.8 mg/kg)
Db (3.6 mg/kg)
Db (0.8 mg/kg) + tPA (10 mg/kg)